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BRIEF COMMUNICATION

Genetic Polymorphisms in *VIR* Genes among Indian *Plasmodium vivax* Populations

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Abstract: The *vir* genes are antigenic genes and are considered to be possible vaccine targets. Since India is highly endemic to *Plasmodium vivax*, we sequenced 5 different *vir* genes and investigated DNA sequence variations in 93 single-clonal *P. vivax* isolates. High variability was observed in all the 5 *vir* genes; the *vir* 1/9 gene was highly diverged across Indian populations. The patterns of genetic diversity do not follow geographical locations, as geographically distant populations were found to be genetically similar. The results in general present complex genetic diversity patterns in India, requiring further in-depth population genetic and functional studies.

Key words: *Plasmodium vivax*, *vir* gene, DNA sequence polymorphism, India

Plasmodium vivax is the most widely spread human malaria parasite and is a major cause of concern in the Central and South America, Central, South and Southeast Asia, India, Middle East, Oceania, and East Africa. Historically, India is highly endemic to *P. vivax* but recent reports suggest that malaria due to *Plasmodium falciparum* and *P. vivax* are in equal proportions [1]. The portrayal of *P. vivax* as benign is now being challenged, as reports of respiratory distress and coma have emerged along with the increasing resistance of the parasite to chloroquine [2-6]. This situation complicates the epidemiology as well as outcome of *P. vivax* malaria not only in India but in global context calling for the urgent attention for effective control measures.

Antigenic genes are present in malaria parasites which encode the variant surface antigens (VSAs) providing protective immunity to the parasite against host/vector. Among these genes, the *vir* family of *P. vivax* is the largest subtelomeric multigene superfamily containing 346 genes divided into 12 subfamilies (A-L) [7,8]. The function of the *vir* gene superfamily is not yet clearly known but they are likely to be involved in antigenic variation and cytoadherence [9,10]. The virulent nature of the VIR proteins leads us to believe that they have a potential role in malaria pathogenesis [7]. Therefore, it is important to study the population genetic diversity of this gene family to understand the recent changing trends of *P. vivax*.

Indian *P. vivax* displays complex evolutionary history and holds several traits of being part of the ancestral distribution range [11]. Not many population genetic studies have been carried out on the *vir* genes of *P. vivax* in India. However, 4 *vir* genes were analyzed for the genetic variability existing in different *P. vivax* populations in India and were found to be highly divergent [12]. In this study, these *vir* genes were further analyzed by population genetic approaches using different statistical tools to facilitate the understanding of the existing diversity of this superfamily among different Indian populations.

In the present study, a total of 191 malaria symptomatic blood samples were collected from patients by finger-prick method. The samples were collected from 6 different epidemiological settings in India, i.e., 39 from Delhi (DEL) in north India, 63 from Mangalore (MNG) in south India, 29 from Goa (GOA) in west India, 39 from Rourkela (RKL) in east India, 9 from Jabalpur (JBL), and 12 from Raipur (RPR) in central India during the years 2008-2011 (Fig. 1).

The ethical clearances for the proposed study to collect blood samples were obtained from the Institutional Ethics Committee, National Institute of Malaria Research (NIMR), New Delhi, India. The patients were briefed about the study verbally and

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Fig. 1. Map of India showing regions where blood samples of malaria patients were collected. The number of *P. falciparum* (*Pf*) and *P. vivax (Pv)* samples collected from each region are shown in the map.

provided a written consent before the samples were collected. Preliminary diagnosis of *P. vivax* was done by microscopy followed by rapid diagnostic tests (RDT) (Bioline SD Rapid Test, San Diego, California, USA). The bloodspots were made on Whatman (no. 3) filter paper strips for further molecular studies and transported to the lab in air-tight sealed bags. Genomic DNA of *P. vivax* was extracted by QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, California, USA) according to the manufacturer's instructions. The isolates were analyzed for mixed infections by *P. falciparum* and *P. vivax* with PCR assays using the published 18s rRNA primers [13]. Furthermore, the single clones of *P. vivax* were determined by genotyping the isolates with merozoite surface protein 3α (*msp3α*) gene.

We followed the traditional PCR protocol using the primers as described earlier for *vir* 27, *vir* 4, *vir* 12, and *vir* 21 [12]. The data generated during the study of these 4 genes have already been published [12]. In this study, we have included 1 more novel *vir* gene (*vir* 1/9) and have reanalyzed the data of the 5 *vir* genes (4 previously published and 1 described in the present study) following different statistical tools to infer diversity patterns among these 5 *vir* genes. The 5 *vir* genes belonged to different subfamilies, i.e., *vir* 27 in subfamily I, *vir* 4 in subfamily C, *vir* 12 in subfamily E, *vir* 21 in subfamily B, and *vir* 1/9 in subfamily J [2]. The *vir* 1/9 gene is 871 bp in length and comprises of 2 exons and 1 intron. Considering the second exon of the *vir* genes to be highly variable [14], we have designed novel primers to amplify the second exon of the *vir* 1/9 gene.

The sequences of the primers are; v1/9_1: 5′ ATGACAAATGGGGGACTCAA 3′ (forward) and v1/9_3: 5′ GAAAATTACTGTTTCCTTAAAATGTGT 3′ (reverse) for the primary PCR

reaction, and v1/9_2: 5′ CGTGAAATGTTATCGGAAAATG 3′ (forward) and v1/9_3 for the semi-nested PCR reaction. The annealing temperature was 50˚C. The purified PCR products were sequenced as described earlier [12]. The accession numbers of the 4 *vir* genes (*vir* 27, *vir* 4, *vir* 12, and *vir* 21) are available in GenBank under accession nos. JQ733915–JQ733988 [12]. The homologous sequences of all the 5 *vir* genes were compared with the Sal-I reference sequences with GenBank nos. (AAKM-01000041.2 for *vir* 27, AAKM01000104.1 for *vir* 4, AAKM-01000016.1 for *vir* 12, AAKM01000003.1 for *vir* 21, and AAKM-01000050.1 for *vir* 1/9).

The sequenced DNA fragments for each *vir* gene were viewed in Finch TV computer program and, edited DNA sequences were then aligned separately to detect single nucleotide polymorphisms (SNPs) with the help of MEGA v 5.10 computer program following the ClustalW algorithm [15]. Minimum evolution phylogenetic tree was constructed for *vir* 1/9 from the sequences of the isolates and Sal-I reference sequence. The computer program DnaSP v 5.10 was used for the sequence analysis of the *vir* genes [16]. For each gene and population sample, the number of segregating sites, number of haplotypes, haplotype diversity, and 2 different measures of nucleotide diversity, π and θ_w, were calculated [17]. Both π and θ_w were used to estimate the extent of nucleotide diversity in a population independently for each of the 5 different *vir* genes. Whereas π measures the average number of pairwise nucleotide differences in a set of DNA sequences, θ_w measures the total number of segregating sites in a set of DNA sequences [18,19]. Tajima's *D* test of neutrality [20] which compares the number of segregating sites per site with the nucleotide diversity was conducted for each gene and each population, and the *D* values were calculated. The direction of Tajima's *D* test can provide useful information about the evolutionary forces that a population has undergone. For example, a negative value of Tajima's *D* highlights an excess of low frequency polymorphisms which shows population size expansion and purifying selection and a positive value signifies low levels of both low and high frequency polymorphisms which shows decreased population size and balancing selection [21]. All the values were considered significant at $P < 0.05$. Furthermore, Pairwise Nei's genetic distances (D) were calculated for the 4 *vir* genes (*vir* 27, *vir* 12, *vir* 21, and *vir* 1/9) independently using GenAlEx v 6.5. For each gene, the population pair-wise genetic distance matrix was used to construct Neighbor-Joining (NJ) phylogenetic trees using the MEGA v 5.10 computer program [22].

Following the preliminary diagnosis by microscopy and RDT of 191 malaria symptomatic patients, only 108 samples were found to be infected with *P. vivax* and the remaining 83 with *P. falciparum*. Further confirmation of differential *P. falciparum* and *P. vivax* infections came from the PCR diagnosis by a nested 18s rRNA PCR assay. However, with PCR assays, 15 isolates from Mangalore were detected as mixed malaria infections with both *P. falciparum* and *P. vivax*. Therefore, we discarded the 15 samples from further analyses. The remaining 93 *P. vivax* single infections were further found to be single clonal with *msp3*α and thereafter analyzed with *vir* specific primers. The distribution of isolates in each Indian population is depicted in Fig. 1.

The amplified sequence lengths for the *vir* genes of the present study ranged between 258-1,314 bp. The sequenced DNA fragments of the 5 *vir* genes were independently aligned (with the respective reference sequences of the SAL-1 strain), manually edited and all the insertions as well as deletions were removed. Multiple sequence alignment of *vir* 1/9 gene showed 78 SNPs out of which only 3 were synonymous mutations showing high diversity in comparison with the Sal-I reference sequence. The NJ phylogenetic tree for 15 samples showed the presence of 2 principal clades. While the first clade comprises of 9 samples (comprising of *P. vivax* isolates from DEL and GOA and the reference isolate), the second clade consists of 7 isolates (with isolates from DEL, GOA, and RKL; Fig. 2). Each clade was further divided into a number of subclades. There was no geographical clustering among the isolates, and the distribution was observed to be randomly presenting very similar profiles as observed in the previous *vir* genes [12]. Comparison of the number of segregating sites among the 5 *vir* genes revealed the presence of as low as 2 SNPs (*vir* 4) to as high as 179 (*vir* 21) among Indian *P. vivax* populations. Among the populations, the sample from GOA had less segregating sites for *vir* 27, *vir* 12, and *vir* 1/9 as compared to the other populations and maximum number of segregating sites for *vir* 21. Similarly, the number of haplotypes varies among the 5 different *vir* genes, with the lowest in the *vir* 4 (3) and highest in *vir* 12 (17). Furthermore, the number of haplotypes of *vir* genes in different populations varies from 1-8 (Tables 1-2). Very similarly, the haplotype diversity was the lowest in *vir* 4 (0.711) and the highest in *vir* 12 (0.962) (Table 1).

The nucleotide diversity parameters as measured by π and θ_w were calculated separately for each gene and population. The average nucleotide diversity parameter π for all 5 genes was

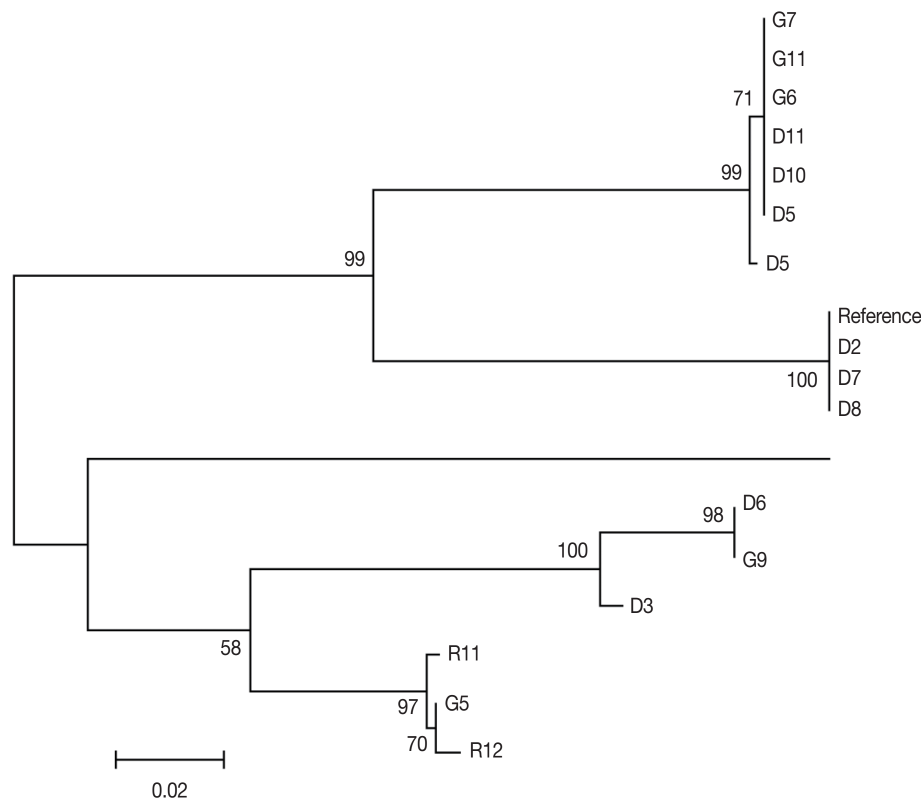

Fig. 2. Phylogenetic tree for *vir* 1/9. The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length=0.53931989 is shown.

Table 1. Genewise summary statistics for the 5 *vir* genes

Vir genes	Sequence length	No. of SNPs (S)	No. of haplotypes	Haplotype diversity	Nucleotide diversity		Tests of neutrality
					π	Θ_w	Tajima's D
Vir 27	1,255 bp	18	15	0.845	0.00265	0.00301	-0.36015
Vir 4	1,314 bp	2	3	0.711	0.00068	0.00054	0.83017
Vir 12	686 bp	120	17	0.962	0.06401	0.0458	1.47617
Vir 21	970 bp	179	13	0.925	0.05557	0.0469	0.70136
Vir 1/9	258 bp	78	9	0.855	0.1128	0.08805	1.07093
Average		79.4	11.4	0.8596	0.047142	0.03686	0.743696

0.047142, and the average value of θ_w was 0.03686 (Table 1). The average value of π was found to be higher than θ_w in *vir* 12, *vir* 21, and *vir* 1/9, but lower in *vir* 27 and almost equal in *vir* 4 gene. The π and θ_w estimates were highest in *vir* 1/9 (π=0.11280 and θ_w=0.08805) and lowest in *vir* 4 (π=0.00068 and θ_w= 0.00054) (Table 1; Fig. 2). Among all the 6 populations, GOA had the lowest nucleotide diversity in *vir* 27 and *vir* 12 genes. In general, the pattern of nucleotide diversities as estimated by π and θ_w were quite variable across the 6 populations of *P. vivax* (Table 1), indicating high diversity among the *vir* genes in Indian populations. Very similar to the estimates of nucleotide diversity, the Tajima's *D* values were quite variable across populations and among the *vir* genes (Tables 1, 2). Wide variations in the Tajima's *D* values therefore indicate ongoing molecular evolution of the *vir* genes in the Indian *P. vivax* populations.

In order to infer genetic interrelationships among Indian population samples of *P. vivax* with respect to 4 *vir* genes sequenced earlier [12], NJ trees were constructed based on the population pair-wise genetic distance matrix (see above). As shown in Fig. 3A-D, the placement of Indian population samples in the NJ phylogenetic tree was different for different *vir* genes. For example, in the NJ trees constructed with the *vir* 27

Table 2. Population wise summary statistics for the 5 *vir* genes analyzed

Region	*Vir* genes	Sequence length	No. of segregating sites (S)	No. of haplotypes	Haplotype diversity	Nucleotide diversity		Tests of neutrality
						π	Θ_w	Tajima's D
Mangalore	*Vir* 27	1,255 bp	13	6	0.833	0.00283	0.00381	-1.22450
	Vir 12	686 bp	101	5	1.000	0.07201	0.07697	-0.48969
Delhi	*Vir* 27	1,255 bp	5	5	1.000	0.00191	0.00191	0.00000
	Vir 12	686 bp	91	4	0.900	0.07172	0.06647	0.59975
	Vir 1/9	258 bp	75	6	0.929	0.12915	0.12407	0.22226
Goa	*Vir* 27	1,255 bp	3	4	0.583	0.00053	0.00088	-1.51297
	Vir 12	686 bp	65	5	1.000	0.05015	0.04828	0.29278
	Vir 21	970 bp	176	7	0.917	0.06446	0.06904	-0.34406
	Vir 1/9	258 bp	52	3	0.700	0.10349	0.09860	0.37405
Rourkela	*Vir* 27	1,255 bp	12	8	0.956	0.00397	0.00338	0.78364
	Vir 12	686 bp	82	3	0.833	0.06487	0.06759	-0.42015
	Vir 21	970 bp	117	4	0.900	0.05515	0.05839	-0.42177

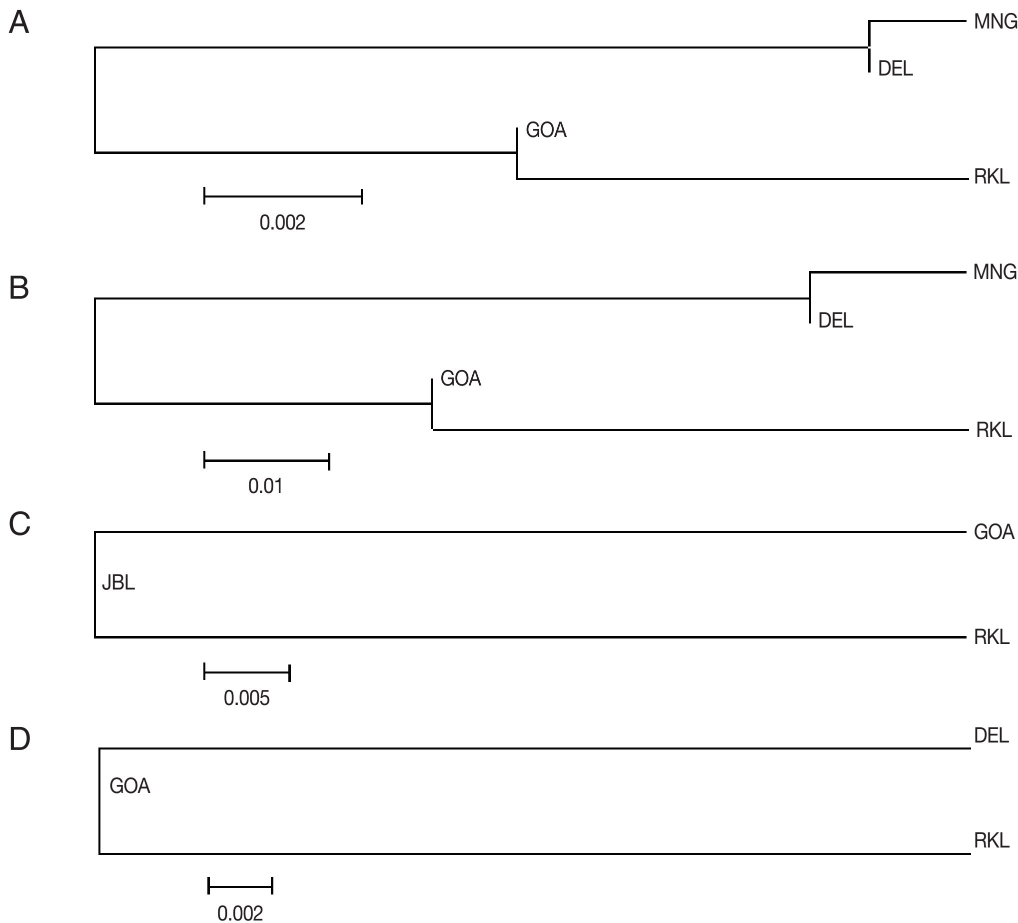

Fig. 3. (A) *Vir* 27 neighbor joining unrooted tree. The phylogenetic tree is based on pairwise Nei genetic distance. The optimal tree with the sum of branch length = 0.01525000 is shown. (B) *Vir* 12 neighbor joining unrooted tree. The phylogenetic tree is based on pairwise Nei genetic distance. The optimal tree with the sum of branch length = 0.08350000 is shown. (C) *Vir* 21 neighbor joining unrooted tree. The phylogenetic tree is based on pairwise Nei genetic distance. The optimal tree with the sum of branch length = 0.05150000 is shown. (D) *Vir* 1/9 neighbor joining unrooted tree. The phylogenetic tree is based on pairwise Nei genetic distance. The optimal tree with the sum of branch length = 0.02750000 is shown.

and *vir* 12 genes, DEL and MNG populations come in 1 cluster and GOA and RKL populations in another, as observed with the *vir* 1/9 gene (see above). The placement of different Indian populations in the NJ phylogenetic tree majorly points towards no particular patterns of genetic relatedness among the Indian populations of *P. vivax*, as for each *vir* gene very different patterns were observed. The results therefore in one hand corroborate the earlier finding on no geographic substructuring of Indian *P. vivax* [7], on the other hand, high sequence diversity of the *vir* genes as previously reported in India [12] has also been found for the *vir* 1/9 gene in India.

It is hypothesized that *vir* genes have a role in malaria pathogenesis [7,23], and *P. vivax* uses the high sequence diversity in these genes to gain high virulence. Therefore, the study on the genetic diversity and evolutionary potentiality of the *vir* genes is essential to understand malaria transmission, disease severity, vaccine development, and various evolutionary aspects of malaria. Population genetic studies further allow us to comprehend the evolutionary history of *P. vivax*, and whether different genes are influenced by natural selection across different geographical regions [21]. Considering that India contributes majorly to the global endemicity of *P. vivax* malaria [1], it is therefore essential to study genetic diversity of *vir* genes in Indian populations. The calculated average nucleotide diversities for the 5 *vir* genes studied here were quite high ($\pi = 0.047142$; $\theta_w = 0.03686$), indicating that Indian *P. vivax* populations maintain high genetic diversity [11]. Interestingly, the GOA population sample contains the least diversity in all aspects of data in comparison to other Indian populations (signifying a more conserved population of *vir* genes) as compared to the other regions of India. Similarly, among the 5 different *vir* genes studied here, the *vir* 4 gene was found to contain the least genetic diversity, indicating this gene might be under the influence of some evolutionary constraint. However, such a conclusion should be taken with caution, as we could sequence a very low number of *P. vivax* isolates for this gene. In contrast, due to the observation of a large number of haplotypes and a high haplotype diversity in *vir* 12, it can be concluded that the *vir* 12 gene is the most diverged gene among the 5 genes studied here.

The NJ phylogenetic trees were constructed from pairwise Nei's genetic distance matix for each of the 4 *vir* genes. Interestingly, the placement of Indian populations was quite different for each of the *vir* genes. For example, in the NJ trees constructed with the *vir* 27 and *vir* 12 genes, DEL and MNG were placed in a single clade and GOA and RKL in another, signifying close genetic affinity between these 2 populations with respect to the 2 *vir* genes (*vir* 27 and *vir* 12). For these 2 genes, GOA and RKL also appear to be genetically identical, as these 2 populations are also placed in a single clade, although geographically these 2 locations are quite wide apart. It is known that genetic relatedness between geographically distant populations can arise owing to the common gene pool shared by the isolates in the past [11]. This phenomenon seems to be common in *P. vivax* populations in India, as very similar patterns of clustering was observed for *vir* 21 as well as in *vir* 1/9 genes. The overall observation in Indian *P. vivax* populations thus revealed that the existing diversity in *vir* genes was randomly distributed without any definite geographic pattern. No observation on any genetic structure among populations with differential malaria endemicity further corroborates this contention. Such genetic epidemiological differences spread across the country may also be responsible for the increased complexity of the *P. vivax* infections which is distinctly exhibited by *vir* genes as observed in this study. This population-based study demonstrates differential levels of diversity in the different geographic regions as also reported in other studies [12]. Observation on the differential calculated values of the Tajima's D also reflect that majorly the Indian *P. vivax* populations present complex demographic history, as observed in an earlier study [11]. Whatever the case may be population genetic studies with more number of *vir* genes and functional analyses will reveal more concrete knowledge on the population evolutionary history [8] of *vir* genes in India.

Due to limited information on the genetic diversity studies in *P. vivax* populations (in comparison to *P. falciparum*), it is difficult to derive concrete conclusion on the observed genetic diversity of the *vir* genes found in the present study. Although several studies on genetic polymorphisms in *P. vivax* have been conducted in worldwide populations [24], genetic diversity studies in Indian *P. vivax* are limited to some antigenic genes [25]. The results from all these studies cannot be compared, as molecular markers are not uniform across countries. Therefore, in order to construct map of global genetic diversity patterns of *vir* genes, similar *vir* genes across *P. vivax* malaria endemic countries are essential. Coupled with functional studies, such a diversity map would inform not only on the extent of extant genetic diversity of *vir* genes, but also will help in designing new vaccines for *P. vivax* malaria management.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest related to this work.

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